Apoptotic Mechanisms After Cerebral Ischemia

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Background and Purpose—Traditionally, cell death after cerebral ischemia was considered to be exclusively necrotic in nature, but research over the past decade has revealed that after a stroke, many neurons in the ischemic penumbra will undergo apoptosis.

Summary of Review—This brief review provides a general overview and update of various signaling pathways in the development of apoptosis in ischemic lesions. Cerebral ischemia triggers two general pathways of apoptosis: the intrinsic pathway, originating from mitochondrial release of cytochrome c and associated stimulation of caspase-3; and the extrinsic pathway, originating from the activation of cell surface death receptors, resulting in the stimulation of caspase-8. Although many of the key apoptotic proteins have been identified, our understanding of the complex underlying mechanisms remains poor and hence treatment of stroke patients by manipulating apoptotic pathways remains a daunting task. However, recent advances in the field have helped broaden our knowledge of apoptosis after cerebral ischemia. Further to the simplistic concept that stroke-induced apoptosis occurs predominantly in neurons and is caspase-dependent, accumulating evidence now indicates that apoptosis is prevalent in nonneuronal cells and that caspase-independent mechanisms also play a key role.

Conclusions—Although the ischemic penumbra is under threat of infarction, it is potentially salvageable and thus represents an opportunity for therapeutic intervention. (Stroke. 2009;40:e331-e339.)

Key Words: stroke ■ penumbra ■ caspases ■ cytochrome c ■ Fas receptor

Within minutes of a focal ischemic stroke occurring, the core of brain tissue exposed to the most dramatic blood flow reduction is fatally injured and subsequently undergoes necrotic cell death. This necrotic core is surrounded by a zone of less severely affected tissue which is rendered functionally silent by reduced blood flow but remains metabolically active. This border region—known as the “ischemic penumbra”—may comprise as much as half the total lesion volume during the initial stages of ischemia, and represents the region in which there is opportunity for salvage via poststroke therapy. Recent research has revealed that many neurons in the ischemic penumbra or perifleref zone may undergo apoptosis after several hours or days, and thus they are potentially recoverable for some time after the onset of stroke. In contrast to necrosis, apoptosis appears to be a relatively orderly process of energy-dependent programmed cell death to dispose of redundant cells. Cells undergoing apoptosis are dismantled from within in an organized way that minimizes damage and disruption to neighboring cells.

There are two general pathways for activation of apoptosis: the intrinsic and extrinsic pathways (see Figure 1). Over the last decade, experimental studies have provided considerable new information characterizing apoptotic processes occurring after ischemic stroke, and an overview of this will be described in this brief review. Differences in responses to ischemia between mature and developing brain have also been described, but this review will focus on experimental findings from studies in mature animals. As targeting and preventing apoptosis in the penumbra would seem to be a rational therapeutic goal for limiting cerebral infarct volume after clinical stroke, the following recent information could provide a basis for design of novel interventions.

Intrinsic Mechanisms of Apoptosis

Channels Mediating Posts ischemic Ca$^{2+}$ Entry

The onset of stroke restricts the delivery of substrates, particularly oxygen and glucose, and impairs the energetics required to maintain neuronal ionic gradients. Rapid depletion of energy after cerebral ischemia leads to a loss of membrane potential and depolarization of nerves. Voltage-dependent Ca$^{2+}$ channels are then activated, and excitatory amino acids are released into the extracellular space. A cytotoxic accumulation of intracellular Ca$^{2+}$ subsequently occurs and is thought to initiate a series of cytoplasmic and nuclear events, including the triggering of the intrinsic apoptotic pathway (Figure 2).

Arguably, the most-studied ion channels in ischemic stroke are the group of receptor operated cation channels opened by glutamate, which accumulates in the extracellular space.
Two types of NMDA receptors are present on neurons, although clinical trials with agents targeting glutamate receptors were disappointing, a possible explanation for those failures of NMDA receptor antagonist-based clinical trials for acute stroke.4,5 Binding of glutamate to ionotropic NMDA (N-methyl-D-aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors promotes a further increase in Ca\(^{2+}\) entry. Interestingly, although clinical trials with agents targeting glutamate receptors were disappointing, a possible explanation for those unfavorable clinical results was recently provided by Liu et al.6 Two types of NMDA receptors are present on neurons, NR2A and NR2B, and Liu and colleagues demonstrated that selective NR2B inhibition prevented NMDA-induced neuronal programmed cell death whereas NR2A receptor blockade failed to inhibit and in fact significantly increased NMDA-induced apoptosis. These surprising observations suggest that activation of NR2A-containing NMDA receptors may trigger cell survival-promoting mechanisms, counteracting the proapoptotic effect of NR2B receptor activation. Hence, the failure of NMDA receptor antagonist-based clinical trials for stroke may have been in part attributable to the “ inadvertent” blockade of “prosurvival” NR2A-NMDA receptors.

The disappointing outcomes from clinical trials of NMDA receptor inhibitors and also L-type Ca\(^{2+}\) channel blockers led to interest in acid-sensing ion channels (ASICs) and transient receptor potential (TRP) channels as possible contributors to apoptosis of neurons after stroke. ASICs are ligand-gated cation channels permeable to Na\(^{+}\), and to a lesser degree Ca\(^{2+}\), which respond to acidic stimuli. It seems likely that these channels play a role in apoptosis induced by cerebral ischemia, in which the degree of acidosis is severe. ASIC1a, the major ASIC subunit with Ca\(^{2+}\) permeability, and also ASIC2a, have been suggested to play a contributory role in cerebral ischemia. Intracerebroventricular administration of the ASIC1a inhibitors, amiloride and psalmotoxin 1, before induction of ischemic stroke, as well as knockout of the ASIC1a gene, is protective. At present, however, this line of evidence is only suggestive, and the specific targets of these drugs remain undetermined. Finally, evidence is emerging that TRP proteins may play a direct role in ischemic stroke, especially in Ca\(^{2+}\)-mediated neuronal death. More specifically, TRPM2 and TRPM7 may be important contributors to the delayed death of neurons after cerebral ischemia. Increasing evidence suggests that these channels potentially contribute to the activation of apoptotic proteins after cerebral ischemia via increased intracellular Ca\(^{2+}\) levels. Conversely, it was recently reported that TRPC3 and TRPC6 are involved in promoting neuronal survival. Whether TRPC channels play a neuroprotective role after cerebral ischemia remains to be delineated.

**Effect of Increased Ca\(^{2+}\) on Mitochondria and the Release of Proapoptotic Proteins**

The role of mitochondria in cerebral ischemic injury remains to be fully elucidated. However, several potentially deleterious mitochondrial responses have been identified after stroke. Examples include the inability to generate ATP and the production of harmful radical species (see below). In addition, mitochondria are thought to be often centrally involved in the development of apoptosis. Mitochondria are considered to influence neuronal apoptosis primarily via the release of proapoptotic factors into the cytoplasm. Activation of calpains by increased Ca\(^{2+}\) or stimulation of caspase-8 via the extrinsic pathway results in the cleaving of Bcl-2 interacting domain (BID) to its truncated active form (tBID). BID is a cytosolic member of the Bcl-2 family of proapoptotic proteins that translocates to mitochondria when the cell receives a death signal. Recent studies have shown that BID is a critical mediator of ischemic cell death within neurons (Figure 2). For example, Plesnila and colleagues found evidence for BID cleavage in ischemic brain tissue and that deletion of the BID gene in mice reduced ischemic infarct size. tBID targets the outer mitochondrial membrane and induces conformational changes in other proapoptotic proteins, such as Bak, Bax, Bad, and Bcl-XL. These proapoptotic proteins can heterodimerise with tBID and antiapoptotic proteins, Bcl-2 or Bcl-XL, via their BH3 domains. In the brain shortly after the onset of ischemia, dephosphorylation and translocation of Bad from the cytosol to the mitochondria occurs and Bad dimerizes with Bcl-XL. Similar observations with Bax have been made in the ischemic brain. The mechanism(s) by which proapoptotic proteins induce the release of apoptogenic factors from the intermembrane space is undetermined, although it is believed to be through the opening of mitochondrial transition pores. Interestingly, it has been suggested that Bach can form channels in liposomes that are large enough to allow passage of cytochrome c.

**Caspase-Dependent Apoptosis**

After the opening of the mitochondrial transition pores, two groups of normally sequestered proapoptotic proteins are released from the intermembrane space into the cytosol. The first group consists of cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi, although we have little knowledge regarding these two latter proteins. Once released, these proteins activate the caspase-dependent mitochondrial pathway. Cytochrome c binds and activates the cytosolic protein Apaf-1 as well as procaspase-9, and together with...
ATP they form an "apoptosome." The clustering of procaspase-9 in this manner leads to caspase-9 activation. Caspase-9, which is presumably an initiator of the cytochrome c-dependent caspase cascade, then activates caspase-3.

Considerable evidence suggests the release of mitochondrial cytochrome c to occur after induction of cerebral ischemia. For example, it has been reported that cytochrome c is translocated from the mitochondria to the cytosolic compartment after either transient or permanent focal cerebral ischemia in rats. Furthermore, immunoprecipitation experiments revealed an enhanced formation of Apaf-1/caspase-9 complex in the hippocampus 8 to 24 hours after global ischemia. These investigators also demonstrated that the specific Apaf-1 inhibitor, Apaf-1 interacting protein, is neuroprotective in cerebral ischemia, and similar observations have also been reported after focal ischemia. Caspase-9 also plays an important role in neuronal death after cerebral ischemia, and Sugawara et al demonstrated cleaved caspase-9 to be increased in brain 12 hours after ischemia. Caspase-3 has been identified as a key mediator of apoptosis in animal models of ischemic stroke. Asahi and coworkers demonstrated upregulation of caspase-3 mRNA in rat brain 1 hour after the onset of focal ischemia. In addition, Namura and associates detected caspase-3 and its cleavage products in mouse brain during early reperfusion after 2-hour middle cerebral artery (MCA) occlusion. Importantly, comparable observations have been extended to ischemic human brain tissue in that caspase-3 was upregulated after ischemia. Both genetic disruption and pharmacological inhibition of caspases have been found to have a strong neuroprotective effect in experimental stroke. Furthermore, cerebral ischemia and reperfusion generate superoxide anions, which causes DNA damage.

Caspase-3 cleaves many substrate proteins, including poly(ADP-ribose) polymerase (PARP). PARP inactivation after cleavage by caspase-3 leads to DNA damage and apoptosis. By contrast, AIF translocates rapidly to the nucleus where it mediates large-scale DNA fragmentation and cell death in a caspase-independent manner. In addition, nuclear pathways of neuronal apoptosis are activated in response to DNA damage, for example, through phosphorylation and activation of p53. Furthermore, cerebral ischemia and reperfusion generate superoxide anions, which causes DNA damage.
mately leads to cellular energy failure and necrotic cell death. Thus, genetic deletion or pharmacological inactivation of PARP reduces cerebral infarct volume after MCA occlusion. Collectively, these findings provide a strong basis for concluding that cerebral ischemia activates the mitochondrial apoptotic pathway, characterized by changes in Bcl-2 family proteins, cytochrome c release, and caspase-like enzyme activation.

**Caspase-Independent Apoptosis**

In addition to cytochrome c/caspase-3-mediated apoptosis, increasing evidence implicates a significant role for caspase-independent pathways in programmed cell death. Such a mechanism includes a second group of proapoptotic proteins released via the mitochondrial transition pores, such as apoptosis inducing factor (AIF), endonuclease G and Bcl-2/adenovirus E1B 19 kDa-interacting protein (BNIP3). Of these proteins, AIF is probably the best understood. There is conclusive evidence that AIF-induced cell death processes are not dependent on functional caspases and could therefore serve as an alternative death pathway after cellular energy depletion that precludes caspase activation. This is supported by findings from Daugas et al., who demonstrated that depletion of ATP acts as a stimulus for AIF release. Once released from the mitochondria, AIF translocates to the nucleus and causes large-scale DNA fragmentation and peripheral condensation of peripheral nuclear chromatin, which is distinct from the global chromatin condensation and oligonucleosomal DNA fragmentation of caspase-dependent death. These differences indicate that AIF causes a unique type of death via a novel intracellular pathway. This raises the question, however, as to whether it remains appropriate to still classify this process as “apoptotic.” Coincidently, some groups have termed it “programmed necrosis” whilst others continue to refer to it as apoptosis.

Caspase-independent neuronal apoptosis has been documented after ischemic stroke. For example, Culmsee and colleagues showed evidence of rapid AIF translocation to the nuclei of injured neurons after cerebral ischemia. In addition, specific inhibition of PARP-1 attenuated the migration of AIF and protected neurons from death after cerebral ischemia. Furthermore, reduced AIF protein levels in siRNA-treated neurons, or in harlequin mutant mice which express low levels of AIF, markedly reduced neuronal cell death after ischemia. Collectively, these data suggest that PARP-1 is an upstream mediator of AIF-induced caspase-independent cell death in ischemic stroke, but the links between PARP-1 activation and AIF release remain to be elucidated. Furthermore, endonuclease G nuclear translocation has been associated with apoptosis after ischemic stroke. Lee et al. demonstrated that endonuclease G becomes detectable in the nucleus 4 to 24 hours after MCA occlusion. Findings from the same study also revealed that AIF and endonuclease G migrate from the mitochondria to the nucleus at about the same time posts ischemia. These observations indicate that endonuclease G may be involved in apoptosis postcerebral ischemia, but the specific nature of the relationship between endonuclease G and AIF has yet to be established. Lastly, BNIP3 (a proapoptotic member of the Bcl-2 family which contributes to mitochondrial dysfunction and cell death) mediates apoptosis independent of both caspases and AIF in primary neuron cultures exposed to hypoxic conditions. This suggests that BNIP3 represents a novel caspase-independent apoptotic pathway that is separate from AIF. Together, the above findings indicate the importance of AIF, endonuclease G, and BNIP3 in caspase-independent cell death after ischemic stroke.

Although the release of cytochrome c and caspase-independent proteins from the mitochondria are a common consequence of the intrinsic apoptotic signaling pathway, there are alternative mechanisms by which mitochondrial dysfunction is likely to contribute to neuronal cell death. Brain cells after cerebral ischemia probably contain damaged mitochondria that are unable to maintain the electrochemical gradient necessary for respiration and glucose oxidation. Indeed a significant reduction in ATP levels in the brain after ischemic episodes is well established. Consequently, these cells may exacerbate cell death by increasing energy failure. Interestingly, because apoptosis is an energy-dependent process, it is possible that intrinsic apoptotic mechanisms may be activated in cells containing energy deficient mitochondria, but there may not be enough energy available to activate further downstream caspases. Hence, treatments that prevent mitochondrial dysfunction, in addition to caspase inhibition, should be considered as a potential protective strategy after cerebral ischemia.

**Reactive Oxygen Species**

Under physiological conditions reactive oxygen species (ROS), which include superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical (OH$^-$), are generated at low levels and play important roles in signaling and metabolic pathways. Intracellular sources of ROS include xanthine oxidase, mitochondrial electron transport chain, arachidonic acid, and NADPH oxidases. Importantly, ROS levels are controlled by endogenous antioxidants such as superoxide dismutases (SOD), glutathione peroxidase, glutathione, and catalase.

Increased levels of ROS are a major cause of tissue injury after cerebral ischemia, in which there is an overproduction of ROS, inactivation of antioxidant enzymes, and consumption of antioxidants, such that natural defense mechanisms fail to protect neurons from oxidative damage. ROS may cause tissue damage after stroke, either directly through participation in destruction of cellular proteins, lipids, and DNA, or indirectly by disrupting normal cellular signaling and gene regulation. Interaction of ROS with other tissue components produces a variety of other radicals. Of particular importance is the interaction of $O_2^-$ with nitric oxide, which produces the highly toxic molecule, peroxynitrite. This oxidant causes further tissue damage and is thought to be an important trigger molecule for apoptosis after ischemic stroke.

It is generally thought that mitochondria are the primary source of ROS involved in ischemia-induced apoptosis. Studies have shown that mitochondrial ROS is produced by a variety of stimuli, including hypoxia itself, excitotoxicity (glutamate), and Ca$^{2+}$ overload after cerebral ischemia. In addition, it has been reported that $O_2^-$ generation is greatest...
after reperfusion, and postischemic reperfusion may particular-  
ycause overproduction of ROS in mitochondria. Once  
generated, mitochondrial ROS influence the release of cyto-  
chrome c and other apoptotic proteins from the mitochondria  
into the neuronal cytosol, which leads to apoptosis and  
defective gene expression after stroke. Thus, increasing  
evidence suggests that oxidative stress and apoptosis are  
closely linked phenomena in the pathophysiology of ischemic  
stroke.

There are numerous reports that ROS derived from  
NADPH oxidase play an important role in various tissues  
after ischemia-reperfusion. Although NADPH oxidase is  
known to be a source of increased $O_2^-$ production after  
cerebral ischemia, studies have yet examined whether  
$O_2^-$ derived from NADPH oxidase activates the apoptotic  
signaling cascade in brain tissue. Another source of increased  
ROS generation that does appear to be related to apoptosis  
after cerebral ischemia is arachidonic acid metabolism.  
Yagami et al demonstrated that phospholipase A$_2$ enzymes  
that release arachidonic acid, known as group IIA secretory  
phospholipase A$_2$ (sPLA2-IIA), induce apoptosis of neurons  
after stroke. This suggests that sPLA2-IIA plays a crucial role  
in apoptosis of neurons in the penumbra of rats after ischemic  
stroke. Overall, the pathway of ROS-induced apoptosis  
represents another potential target for poststroke therapy.

DNA Damage

DNA damage, if irreparable, may activate apoptotic signaling  
mechanisms. In response to DNA damage, the tumor-  
suppressor transcription factor, p53, stops the cell cycle and  
triggers programmed cell death by promoting proapoptotic  
protein expression, suppressing antipapoptotic protein  
regulation, and targeting BH3-only proteins, p53-upregulated  
modulator of apoptosis (PUMA) and NOXA. PUMA can inhibit  
all the antipapoptotic proteins and activate the intrinsic death  
pathway by switching on proapoptotic proteins, such as Bax  
and Bak. PUMA is activated at the transcriptional level by  
p53 and is induced by all stimuli that activate p53, including  
DNA damage and oxidative stress. Similarly, studies have  
shown that NOXA can translocate to the mitochondria and  
interact with antipapoptotic Bcl-2 family members, resulting  
in the activation of caspases. Furthermore, it has been reported  
that p53 activates transcription of the Fas receptor after DNA  
damage, which raises the surprising notion that nDNA  
damage signals can be transduced via cell surface receptors.  
It is interesting to note, however, that early p53-induced  
apoptotic cells can be rescued from the apoptotic program if  
the stimulus is removed. Research by Geske and coworkers  
suggested that DNA repair is activated early in the  
p53-induced apoptotic process and may be able to reverse the  
cell death pathway.

p53 is a promising target for stroke therapy, as it is rapidly  
upregulated in ischemic brain tissue where it initiates program-  
med cell death through the transcription of Bax, PUMA,  
or NOXA, as well as the subsequent mitochondrial damage  
and activation of caspases. For example, Endo and col-  
leagues reported transcription-independent proapoptotic  
activity of p53 in a rat model of transient global ischemia, in  
which cell death occurred in hippocampal CA1 neurons after  
the mitochondrial translocation of p53 and its interaction with  
the antia apoptotic Bcl-xL. In addition, it has been identified  
that PUMA expression is upregulated in regions of the  
hippocampus known to exhibit significant levels of apoptosis  
after cerebral ischemia, suggesting that PUMA could be a key  
target for poststroke therapy. Interestingly, harmful DNA  
damage-dependent signaling events, including p53 activation,  
and mitochondrial translocation of PUMA and NOXA, were  
reduced during postischemic reperfusion in hyperthermia-  
treated rat brains. These findings suggested that DNA  
damage-triggered prodeath signaling events may be an  
important mechanism underlying the neuroprotective effect of  
mild hypothermia against ischemic brain injury.

Extrinsic Mechanisms of Apoptosis

Fas and TNF Receptor

Extrinsic mechanisms of apoptosis involve the engagement of  
death receptors located on the plasma membrane and is hence  
also referred as the “death receptor pathway” (Figure 3). Cell  
surface death receptors belong to the tumor necrosis factor  
receptor (TNFR) superfamily, and include TNFR-1, Fas, and  
p75NTR. Forkhead1, a member of the forkhead family of  
transcription factors, stimulates expression of target genes,  
such as the Fas ligand (FasL), which are implicated in the  
extrinsic receptor pathway of caspase-3 activation. FasL  
initiates apoptosis by binding to the Fas receptor, triggering  
recruitment of the cytoplasmic adaptor protein Fas-associated  
death domain protein (FADD). FADD contains a “death  
effector domain” at the N terminus which binds to  
procaspase-8 by interacting with its death effector domain.  
This complex (FasL–Fas–FADD–procaspase-8) is referred as  
death-inducing signaling complex (DISC) and is assembled  
within seconds of Fas receptor engagement. The signal  
complex catalyzes the proteolytic cleavage and transactiva-  
tion of procaspase-8 to generate caspase-8. Once activated,  
caspase-8 is released from the DISC complex into the  
cytoplasm and initiates downstream cleavage of caspase-3 by  
direct or mitochondrial-dependent mechanisms, thus paving  
the way for the execution phase of apoptosis involving PARP  
cleavage, as discussed above.

There is now considerable evidence that cerebral ischemia  
triggers the extrinsic apoptotic signaling cascade. Fas, FasL,  
and TNF-related apoptosis-inducing ligand expression are  
upregulated in the posts ischemic rat brain. Interestingly,  
this upregulation was observed within 12 hours after cerebral  
ischemia and peaked between 24 and 48 hours, which  
coincides with the time course of apoptotic death in neuronal  
cells. Most importantly, mice expressing dysfunctional  
FasL, and also TNF knockout mice, have significantly  
reduced cerebral infarcts after MCA occlusion. Remarkably,  
hybrid mice lacking both cytokines had a 93% reduction in  
infarct volume 24 hours after stroke. A similarly profound  
level of protection was observed in wild-type mice treated  
with neutralizing antibodies against both FasL and TNF.  
These findings emphasize the contribution of death receptors  
in cell death after cerebral ischemia.
Apoptosis in Nonneuronal Cells

Our initial knowledge of apoptosis after cerebral ischemia was largely confined to its occurrence in neurons as early studies reported that almost all of the apoptotic cells in the ischemia core were identified as neuronal. However, there has been a steady increase in evidence relating to apoptosis in nonneuronal cells within the brain after stroke. For example, apoptosis of glial cells and infiltrating leukocytes after brain ischemia has been observed in some experimental models of stroke. Upregulation of caspase-3 and several other caspase family members has been reported in astrocytes and microglia after MCA occlusion. Furthermore, Chen et al identified DNA strand breakage in astrocytes in the border zone of the infarcted tissue after cerebral ischemia. However, the extent of ischemia-induced programmed cell death in glial cells remains uncertain and further research is required.

Similarly, little is known about programmed cell death in vascular tissue, although a small number of studies have examined apoptotic signaling mechanisms in vascular endothelial cells after ischemia-reperfusion. Diminished cerebral blood flow after cerebral ischemia triggers a series of processes that affects the microvasculature by disrupting the blood-brain barrier integrity and the regulation of arterial tone. As discussed above, large amounts of ROS, especially $O_2^-$, are generated in the brain after reperfusion, and ROS have been reported to activate apoptosis in vascular endothelial cells. In addition to the detrimental vascular actions of $O_2^-$ in decreasing blood–brain barrier integrity and increasing vascular tone as mentioned above, its reaction with endothelium-derived nitric oxide to form the highly toxic peroxynitrite radical would be expected to trigger apoptosis in vascular cells after ischemic stroke. Evidence for vascular apoptosis has also been reported after subarachnoid hemorrhage in endothelial cells of basilar arteries, in which double fluorescence labeling demonstrated colocalisation of TUNEL, which detects DNA fragmentation, with either caspase-3, caspase-8 or TNFR1. These observations suggest that the mechanism of apoptosis in cerebral endothelial cells after subarachnoid hemorrhage involves TNFR1 and the caspase-8 and caspase-3 pathways. There is now a need to characterize the features of apoptosis in cerebral vascular cells after ischemic stroke. It is too early to predict whether specific or additional targeting of the vasculature to prevent apoptosis might represent a useful strategy for poststroke therapy.

Endogenous Antiapoptotic Mechanisms and Their Potential as Therapeutic Targets

Interactions between the proapoptotic and antiapoptotic Bcl-2 family proteins on the outer mitochondrial membrane are
believed to play an important role in cell survival. Typi-
cally, antiapoptotic members are located on the outer mito-
chondria membrane and suppress apoptosis through multiple
mechanisms, including conserving mitochondrial membrane
potential, inhibiting the release of apoptotic proteins, stabi-
lizing the mitochondrial transition pores, thus preventing its
opening, and controlling the activation of caspase proteases.

Bcl-2 and Bcl-xl levels are downregulated within the first
few hours of cerebral ischemia. Interestingly, significantly
smaller infarcts have been observed in mice overexpressing
Bcl-2 or Bcl-xl after focal cerebral ischemia whereas stroke
in Bcl-2 knockout resulted in an increased infarct size. Studies
have demonstrated that various neuroprotective
treatments reduce the impact of stroke via increasing
expression levels of either Bcl-2 or Bcl-xl. For example, the
administration of a Bcl-xl fusion protein that contains the
TAT domain of the human immunodeficiency virus is pro-
tective in models of ischemia. Furthermore, estradiol exerts
profound protective effects on ischemic brain tissue and
prevents the ischemic-induced downregulation of Bcl-2 ex-
pression, indicating that estradiol modulates the expression
of Bcl-2 as a neuroprotective mechanism. Collectively,
these findings suggest the importance of antiapoptotic Bcl-2 family
proteins in ischemic cerebral injury, and thus therapeutics
targeting these proteins may potentially provide neuroprotec-
tion against stroke.

It is important to note, however, that the neuroprotective
effects of Bcl-2 and Bcl-xl can be neutralized through heterodimerization with proapoptotic proteins, such as Bad, which subsequently promotes cell death. Nevertheless, various upstream signaling mechanisms can stimulate tran-
scription of antiapoptotic proteins or suppress the inhibitory
effect of Bad on Bcl-xl. For example, phosphoinositide
3-kinase phosphorylates Akt, which subsequently phosphor-
ylates Bad and eliminates its inhibitory effects on Bcl-xl in
neurons after ischemia. Furthermore, extracellular signal-
regulated kinase 1/2 (ERK1/2) inactivates Bad through phos-
phorylation of 90-kDa ribosomal S6 kinases. Indeed, trans-
forming growth factor-β1 has been reported to phosphorylate
and inhibit Bad activity via activation of the ERK pathway
in brain during ischemia. Consistent with those observations,
Kilik and coworkers using a transgenic mouse that ex-
presses elevated levels of the human hematopoietic growth
factor, erythropoietin, in the brain demonstrated that erythro-
poietin protects against focal cerebral ischemia via activating
ERK1/2 and Akt signaling. In the same study, Bcl-xl
expression in ischemic brain tissue was elevated above the
levels in wild-type mice, suggesting that ERK1/2, Akt, and
Bcl-xl pathways play a role in the neuroprotective function of
erythropoietin. Protein kinase A (PKA) can also phos-
phorylate Bad after cerebral ischemia, thus triggering its
association from Bcl-xl. The cumulative evidence present-
ed above indicates that Akt, ERK1/2, and PKA pathways
inhibit Bad function as well as triggering prosurvival signal-
ing pathways after cerebral ischemia.

Lastly, another family of regulatory proteins are the endoge-
nous inhibitors of apoptosis (IAPs), which act by inhibiting
caspases-3 and -9. Levels of X-linked IAP and the number of
XIAP-positive cells show a strictly time- and region-dependent
evolution in cerebral ischemia, suggesting that this IAP plays a
role in regulation of apoptosis in this setting. Transgenic mice
overexpressing human XIAP undergo reduced caspase-3 activa-
tion after stroke and thus have reduced brain damage.

**Conclusions**

It has become increasingly clear that there are many complex
signaling pathways involved in apoptosis after cerebral ische-
mia. Although some inroads have been made in identifying
specific molecular pathways involved in apoptosis after stroke,
the long-term we will require an integrated understanding of
the multifaceted molecular processes that contribute to apoptosis
to identify relevant novel stroke therapies.

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